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The antiproliferative effect of C₂-ceramide on lung cancer cells through apoptosis by inhibiting Akt and NFκB

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Abstract

The anticancer effects of ceramide have been reported in many types of cancers but less in lung cancer. In this study, we used C₂-ceramide to further investigate its possible anticancer effects and mechanisms on non-small cell lung cancer (NSCLC) H1299 cells. The result of cell proliferation in terms of trypan blue assay showed high dose of C₂-ceramide inhibited cell survival after 24 h treatment. The flow cytometry-based assays indicated the effect of apoptosis, chromatin condensation, and G₁ arrest in terms of Annexin V/propidium iodide (PI), DAPI, and PI stainings, respectively. Moreover, the decreased protein level of p-Akt, p-NFκB, survivin and cyclin A2 were detected by Western blot assay. Taken together, these results indicated the antiproliferative effect of C₂-ceramide is majorly responsible for cell apoptosis in lung cancer H1299 cells.

Keywords: Lung cancer, Apoptosis, NSCLC, Ceramides, p-Akt, p-NFκB, survivin, cyclin A2

Introduction

Approximately 80% of lung cancer belongs to non-small-cell lung cancer (NSCLC) and the other is SCLC histologically [1]. Although smoking is one of the main risk factors for lung cancer [2], about 10% patients are non-smokers [3]. NSCLC is generally detected and diagnosed at late stage and its prognosis is poor [4]. Therefore, the anticancer drugs for NSCLC treatment remain a challenge.

Akt, a serine/threonine kinase, regulates cell growth, cell cycle progression, survival and anti-apoptosis. Dysregulation of Akt was reported to be observed in various cancers including breast cancer [5] and lung cancer cells [6]. Furthermore, the constitutive activation of Akt has been shown to cause chemoresistant of cancer cells. Similarly, NFκB, an inflammatory-associated transcription factor, is also found to be constitutively activated

or aberrantly expressed in lung cancer [7,8]. Therefore, targeting of Akt and NFκB signaling seems to be a promising strategy for lung cancer therapy [9-12].

Ceramides are important components composed of lipid molecules and can form into sphingolipids when added functional groups on their hydroxyl group such as phosphate choline or monosaccharide. When cells are triggered by certain stimuli, an enzyme called sphingomyelinase [13] would hydrolyze sphingolipids and cause the release of ceramides into cytoplasm, which can undergo many biological processes, such as differentiation, proliferation, growth arrest and apoptosis [14]. Ceramide was reported to act as an important mediator in apoptosis pathways. Exogenous ceramides have been demonstrated to induce anti-proliferation and apoptosis in many cancers [15]. Furthermore, accumulating evidence showed that ceramide inhibits proliferation of cancer cells via inhibiting Akt and NFκB signal pathways [16,17].

The ceramide-mediated anticancer effects have been reported in many types of cancers such as pancreatic [18], breast [19], gastric [20], hematologic [21] cancer. However, the final outcome of ceramide treatment may depend on the context of cell types. Previous studies

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showed that knockout of *NFκB p65* sensitizes embryonic fibroblasts toward C_2 -ceramide induced cell death [22]. On the contrary, the above study also found that C_2 -ceramide induces cell death and activation of NFκB in lung cancer H1299 cells [22]. The effects of C_2 -ceramide on apoptosis of H1299 cells were investigated previously [22,23]. In the current study, we examined the growth inhibitory property to NSCLC H1299 cells by C_2 -ceramide as well as its possible apoptosis mechanism, especially inhibiting Akt and NFκB pathways.

Materials and methods

Cell cultures

The H1299 lung cancer cells were maintained in DMEM medium (Invitrogen, Carlsbad, CA USA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.03% glutamine and 1 mM sodium pyruvate [24] and kept at 37°C in a humidified atmosphere with 5% CO₂.

Cell survival assay

Cell survival was determined by the trypan blue dye exclusion assay as previously described [25,26]. In brief, Cells were seeded at a density of 1×10^5 cells per well. After 24 h of incubation, the cells were treated with C_2 -ceramide (#A7191, Sigma) at concentrations of 0, 10, 20, and 50 µM for 24 h, then 0.2% trypan blue were added to wells. Finally, the viable cells we are calculated by the Countess® Automated Cell Counter (Invitrogen, Diego, CA, USA). The assay was triplicated and the IC₅₀ was calculated by the slope and intercept accordingly to two concentrations of C_2 -ceramide between the half-maximal proliferative inhibition.

Apoptosis assay

Apoptosis was detected by annexin/PI staining (Pharmingen, San Diego, CA, USA) as previously described [27]. Briefly, cells were treated with C_2 -ceramide at concentrations of 0, 10, 20, and 50 µM for 24 h. After collection, cells were treated with 10 µg/ml of annexin V-fluorescein isothiocyanate and 5 µg/ml of PI for analysis with a FACSCalibur™ flow cytometer (Becton-Dickinson).

Chromatin condensation assay

5×10^5 H1299 cells were seeded onto a 6-well plate. After 24 h, cells were treated with indicated concentrations (0 to 50 µM) of ceramide for 24 h. After wards, cells were stained with 5 µg/ml of DAPI for 3 mins at 37°C. The level of chromatin condensation was determined by a flow cytometry (Becton-Dickinson). At least 10,000 stained cells were counted and calculated as percentage of chromatin condensation compared to those of the control cells.

Cell cycle distribution

Propidium iodide (PI, Sigma, St. Louis, MO, USA) staining for DNA content measurement was performed as described previously [28]. Briefly, cells were treated with 0, 10, 20, and 50 µM of C_2 -ceramide for 24 h. After collection, cells were washed twice with PBS before 70% ethanol fixation. After centrifugation, the cells were incubated with 10 µg/ml PI and 10 µg/ml RNase A in PBS for 15 min at room temperature in the dark. Cell cycle analyses were performed using a FACSCalibur flowcytometer (Becton-Dickinson, Mansfield, MA, USA).

Western blotting

Western blot assay was performed as described previously [27]. Briefly, cells were collected for lysate preparation. After centrifugation, and protein concentrations of lysates were determined. Protein lysates for 40 µg were loaded and electrophoresed by 10% SDS-polyacrylamide gel (PAGE) and then transferred to membrane. The membranes were blocked with 5% non-fat milk. Subsequently, it was reacted with primary antibodies against t-Akt (#1081), p-NFκB (Ser536, #2220) and Bax (#1063, Epitomics, CA, USA); t-NFκB (sc-8008), β-catenin (sc-7963) and p-Akt (Ser473, sc-7985, Santa Cruz Biotech, Santa Cruz, CA, USA); Cyclin A2 (GeneTex Co., Cat No. GTX103042); survivin (AnaSpec, San Jose, CA, USA) and β-actin (#sc-8432, Santa Cruz Biotech), and their corresponding secondary antibodies. The ECL™ (Amersham Piscataway, NJ, USA) chemiluminescence detection kit was applied.

Statistical analysis

All data are presented as mean ± S.D. Comparison between experimental groups and vehicle controls was assessed by one-way ANOVA test.

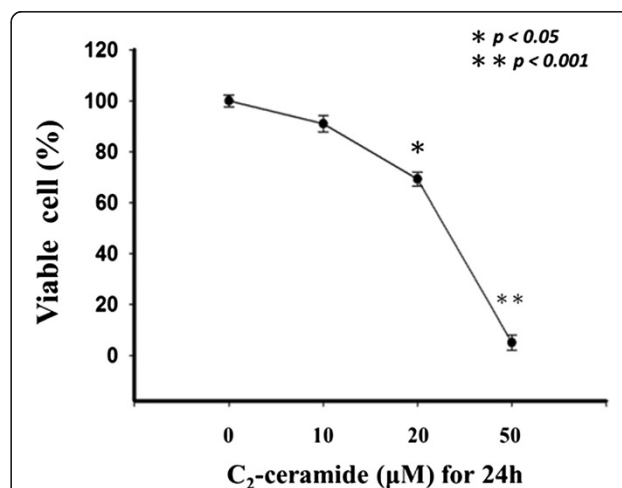
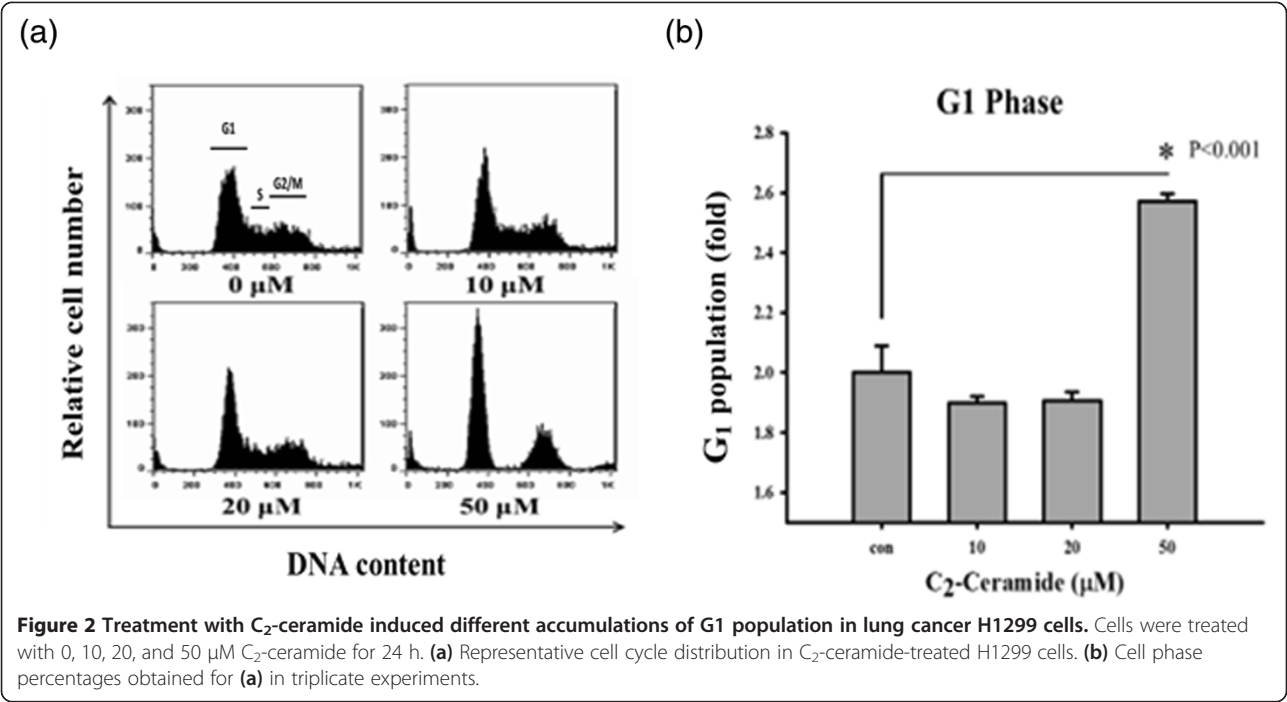


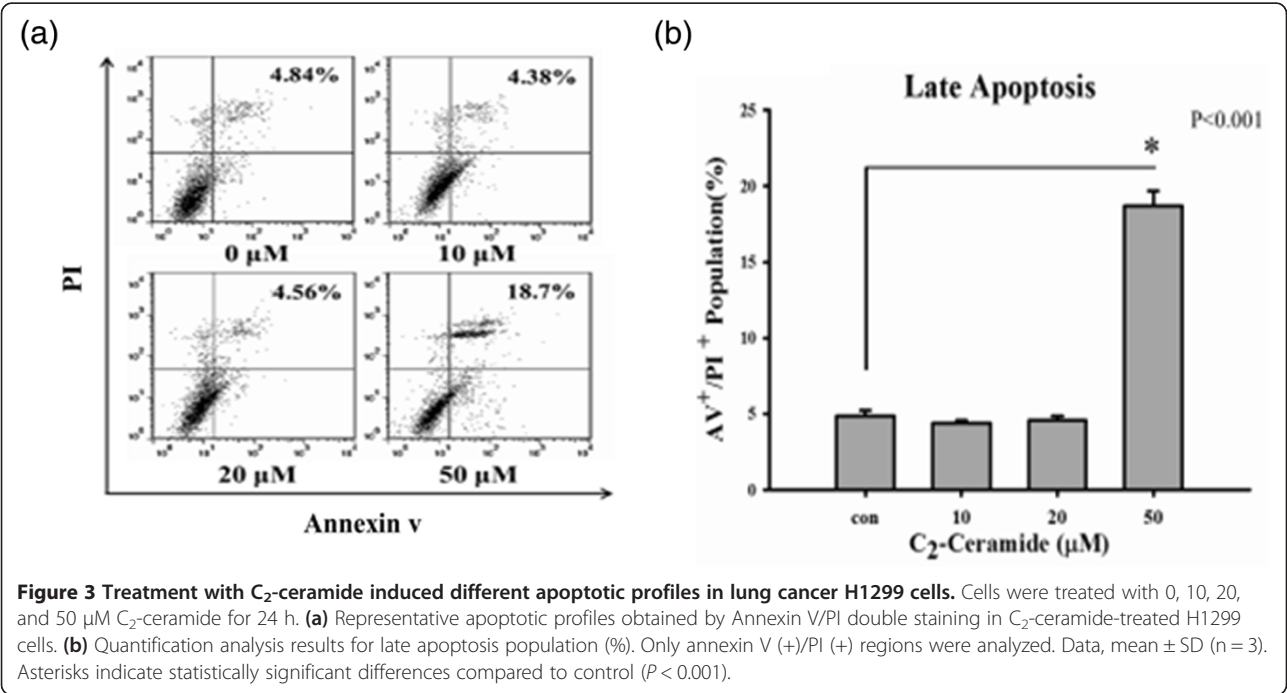
Figure 1 Effect of C_2 -Ceramide on proliferation of H1299 cells.
The cell proliferation assay showed the inhibition of cell growth at high dose of treatment for 24 h.

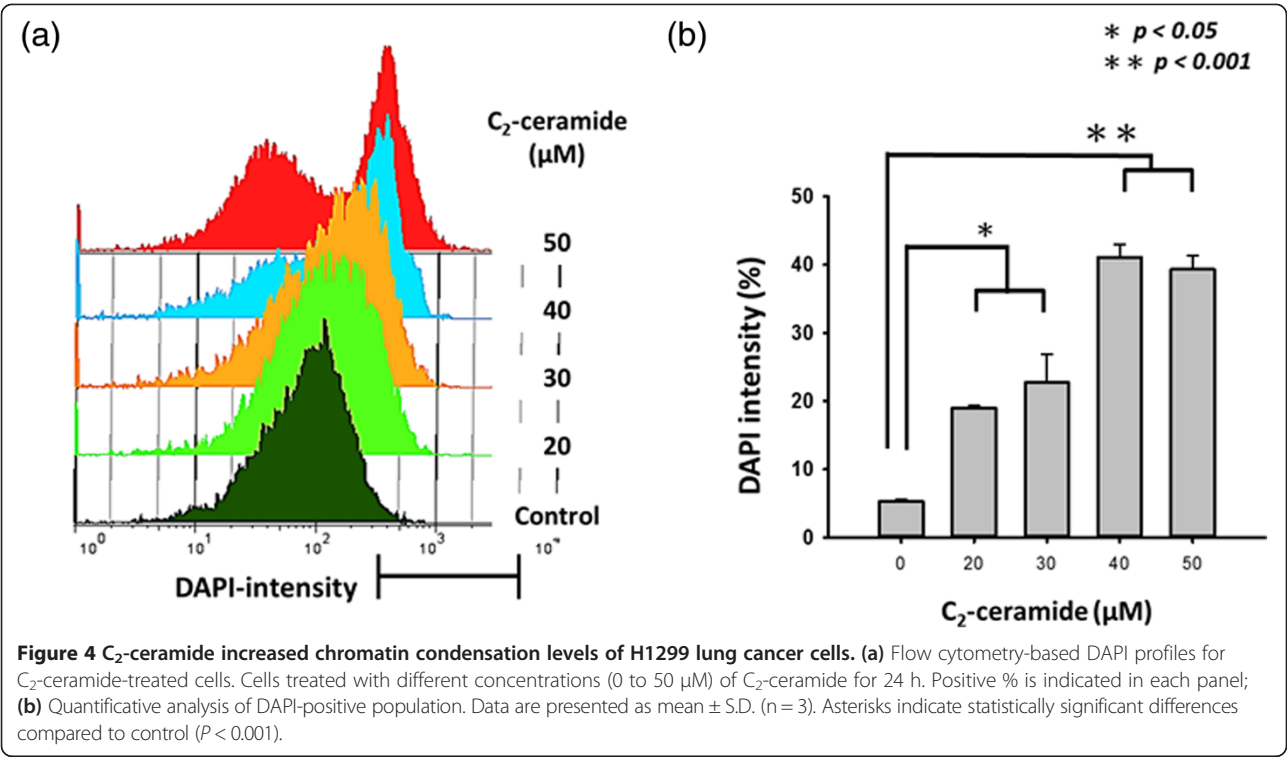


Results
Anti-proliferative effects of C₂-Ceramide-Treated H1299 lung cancer cells
In the trypan blue assay (Figure 1), the proliferation rates at various concentrations of C₂-ceramide (0, 10, 20, and 50 μM) after 24 h were 100.0 ± 2.3, 90.1 ± 3.2, 69.2 ± 2.8, 5.0 ± 3 (n = 3). The proliferation rate of C₂-ceramide-treated H1299 lung cancer cells significantly decreased in

a dose–response manner (P < 0.001). The 50% inhibitory concentration (24 h, IC₅₀) of C₂-ceramide for H1299 cells was 22.9 μM.

G₁ arrest of C₂-Ceramide-treated H1299 lung cancer cells
The role of cell cycle interference in the C₂-ceramide-induced apoptosis of H1299 lung cancer cells was examined by the flow cytometry-based PI assay (Figure 2).

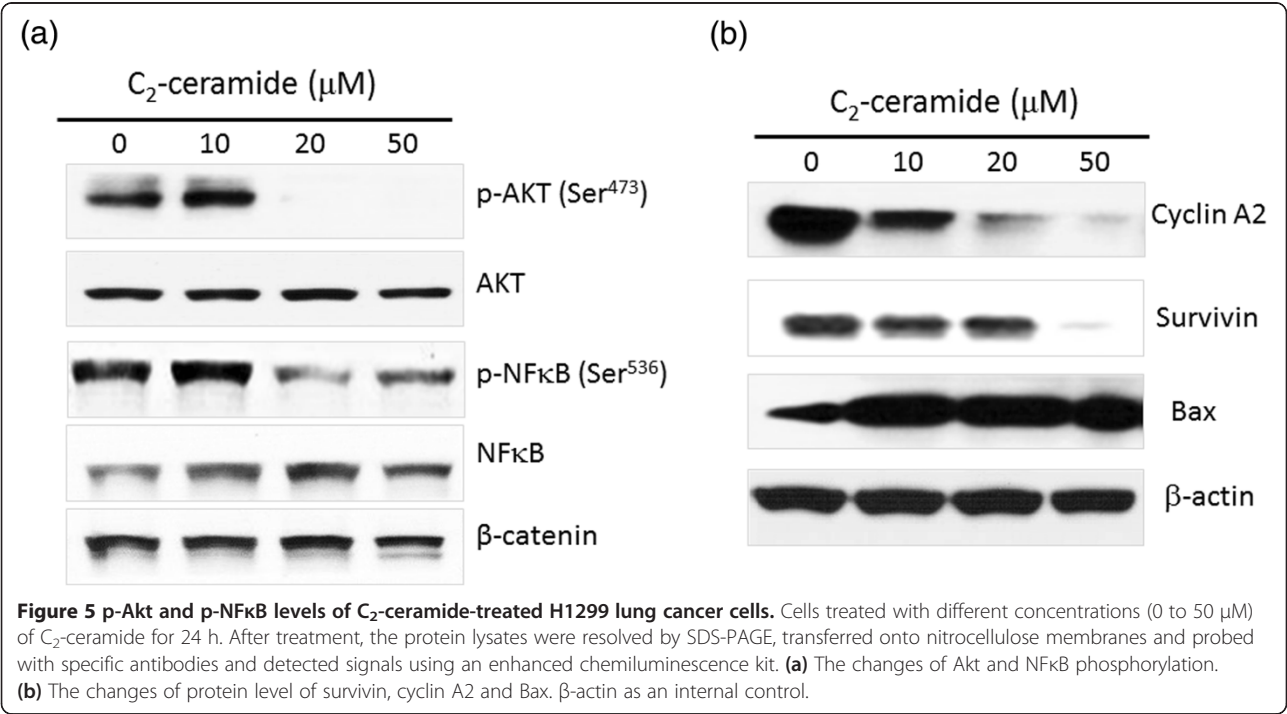




The G₁ percentages were increased at concentration of 50 μM C₂-ceramide for 24 h (Figure 2a). Apparently, the G₁ arrest activities in cells treated with C₂-ceramide showed a significant increase at higher concentration ($P < 0.001$) (Figure 2b).

Apoptosis induction of C₂-Ceramide-treated H1299 lung cancer cells

In Figure 2a, the profiles of PI/annexin V-positive percentages were shown for the treatments with vehicle control or 0, 10, 20, and 50 μM of C₂-ceramide for 24 h. After 24 h



C₂-ceramide treatment, the annexin V-positive percentages of H1299 lung cancer cells were significantly increased in a dose-response manner for most concentrations ($P < 0.05$) (Figure 3).

Chromatin condensation of C₂-Ceramide-treated H1299 lung cancer cells

Chromatin condensation is one of the most important markers for apoptotic cells [29]. The apoptotic effect of C₂-ceramide-treated H1299 lung cancer cells was further examined by the flow cytometry-based DAPI assay. The profiles of DAPI-positive percentages of 0, 10, 20, and 50 μ M C₂-ceramide for 24 h were shown (Figure 4a). The DAPI-positive percentages of C₂-ceramide-treated H1299 lung cancer cells were significantly reduced in a dose-response manner ($P < 0.001$) (Figure 4b).

Modulation of p-Akt and p-NF κ B in C₂-Ceramide-treated H1299 lung cancer cells

The role of C₂-Ceramide-induced modulating the levels of p-Akt and p-NF κ B in H1299 lung cancer cells was examined by Western blotting. Both p-Akt and p-NF κ B levels in C₂-Ceramide-treated H1299 cells were significantly reduced at the concentration of 20 and 50 μ M (Figure 5a). Likewise, the protein levels of pro-survival survivin and the cell cycle promoter cyclin A2 were down-regulated dramatically. On the contrary, the protein level of pro-apoptotic Bax was increased significantly following C₂-Ceramide treatment (Figure 5b).

Discussion

The modulations of ceramide as the strategy for many kinds of cancer therapies have been reported [18-21]. For example, acid ceramidase was regarded as the target for breast cancer therapy [19] because it can hydrolyze ceramide, and thus reduce its intracellular levels. Previous study showed that C₂-ceramide is not very harmful to normal cells. For example, the IC₅₀ (24 h) of human dermal neonatal fibroblast (HDNF) cells for C₂-ceramide was 66.5 μ M [30], suggesting the moderately selective anti-proliferative effect of C₂-ceramide toward cancer cells. In the current study, we found that the C₂-ceramide induced apoptosis of H1299 lung cancer cells. It provides the idea that pharmacological modulation of sphingolipid metabolism can enrich the tumor cell ceramide for cancer chemotherapy.

Sometimes the degree of the sub-G₁ accumulation may not appear in concert with the apoptosis in terms of Annexin V/PI staining. For example, no sub-G₁ accumulation was found in C₂-ceramide-treated H1299 lung cancer cells at 24 h treatment, but it still showed the apoptosis-inducible effects in terms of Annexin V/PI and DAPI-based chromatin condensation assays using flow cytometry. Similarly, (-)-Anonaine inhibits growth

of H1299 cells without sub-G₁ accumulation before 48 h incubation, however, the (-)-Anonaine ends up increasing apoptosis at 72 h treatment [31]. Therefore, the absence of sub-G₁ accumulation in C₂-ceramide-treated H1299 at 24 h treatment may be due to the detection timing. Furthermore, chromatin condensation was thought to be one of hallmarks in apoptotic cells [32,33]. However, some study indicated that certain stresses such as heat shock may induce a non-apoptotic chromosome condensation [34]. For example, Plehn-Dujowich's work found the non-apoptotic chromatin condensation [34]. Accordingly, in our study, the non-apoptosis inducing dose of 20 μ M C₂-ceramide caused stress-induced chromatin condensation, which may explain the reason that C₂-ceramide induces anti-proliferation without apoptosis (Figure 4). Eventually, a higher dose of 50 μ M C₂-ceramide causes the apoptotic chromatin condensation, resulting in cell death of H1299 cells.

Previously, C₂-ceramide-induced H1299 cells was investigated [22,23]. Demarchi's work indicated that C₂-ceramide triggers the NF κ B-dependent survival pathway.

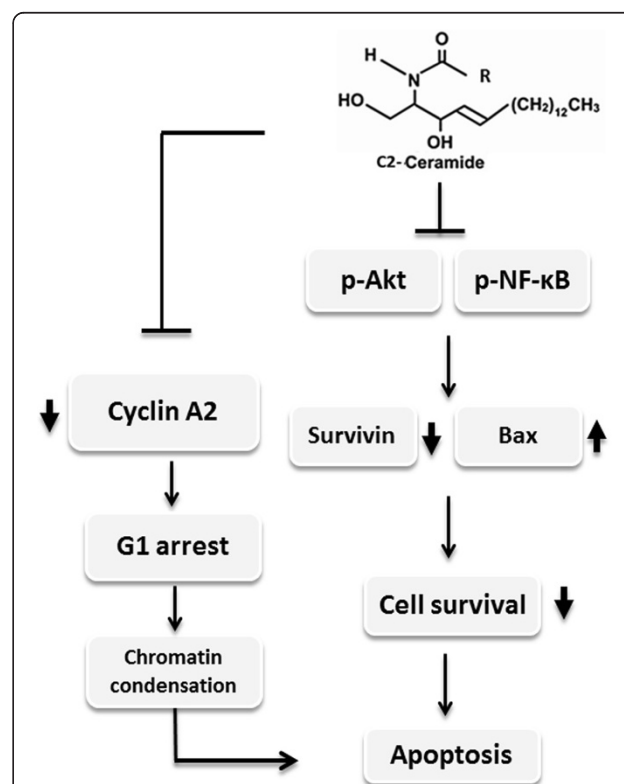


Figure 6 Schematic diagram of hypothesized mechanism of C₂-ceramide-induced apoptosis of lung cancer cells. C₂-ceramide inhibits the activity of both Akt and NF- κ B, causing the down-regulation of pro-survival survivin and cell cycle promoter cyclin A2. On the contrary, C₂-ceramide increases the protein level of pro-apoptotic Bax. As a result, C₂-ceramide treatment causes cell cycle G₁ arrest and chromatin condensation, subsequently, triggering the apoptosis of lung cancer cells.

However, our study showed that C₂-ceramide dramatically decreases the level of phosphorylated NFκB (Figure 5a). This may due to the different duration of NFκB treatment (8 h vs. 24 h for Demarchi and Lin respectively). Importantly, our study demonstrated that C₂-ceramide potently inhibits Akt phosphorylation of H1299 cells at the of 20 and 50 μM, suggesting that it will be an advantage of treating lung cancer with constitutively phosphorylated Akt. C₂-ceramide also causes the down-regulation of survivin and cyclin A2 (Figure 5b), and the up-regulation of pro-apoptotic factor Bax in H1299 cells. This may sensitize lung cancer cells towards proliferation inhibition and apoptosis (Figure 6). The results of this study demonstrated that that C₂-ceramide treatment exerts anti-growth potential against human non-small cell lung cancer cells H1299 in a dose-responsive manner. C₂-ceramide also reduces the pro-survival proteins Akt and NFκB, causing the down-regulation of survivin and cyclin A2, which are reported to frequently overexpress in non-small cell lung cancer [35]. This may sensitize lung cancer cells towards proliferation inhibition and apoptosis (Figure 6). Accordingly, the above results suggested that C₂-ceramide may be a promising reagent for lung cancer treatment or adjuvant therapy in future.

Competing interest

The authors declare no conflict of interest.

Author's contribution

Conceived and designed the experiments: HLC, HMW and ILL; Performed experiments: HLC, JCL and FWC; Data analysis: YH and HWH; Contributed reagents/materials: WCC, CYW and WTC; Manuscript writing: CCC and ILL. All authors have read and approved the manuscript.

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